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<b>(21) International Application Number:</b> PCT/GB94/02180 <b>(22) International Filing Date:</b> 6 October 1994 (06.10.94)  <b>(30) Priority Data:</b> 9320782.7 8 October 1993 (08.10.93) GB  <b>(71) Applicant (for all designated States except US):</b> THE UNIVERSITY OF LEEDS INNOVATIONS LTD. [GB/GB]; 175 Woodhouse Lane, Leeds LS2 3AR (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GIBSON, Timothy, David [GB/GB]; 61 High Ridge Park, Rothwell, Leeds LS26 0NL (GB). PIERCE, Barry, Leigh, John [GB/GB]; 15 Regents Park Avenue, Leeds LS6 1AU (GB). WEBSTER, Jeanette, Irene [GB/GB]; 21 Elm Place, Ormskirk, Lancashire L39 4TF (GB).  <b>(74) Agent:</b> BROWNE, Robin, Forsythe; Urquhart-Dykes & Lord, Tower House, Merriion Way, Leeds LS2 8PA (GB).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> STABILISATION OF PROTEINS IN SOLUTION  <b>(57) Abstract</b>  A protein stabiliser additive comprises two or more of a tris compound of the formula (1): $(\text{HOCH}_2)_3\text{-C-R}$ , wherein R is: C <sub>1</sub> -C <sub>4</sub> alkyl, substituted C <sub>1</sub> -C <sub>4</sub> alkyl, NH <sub>2</sub> ; NR <sup>1</sup> R <sup>2</sup> wherein R <sup>1</sup> and R <sup>2</sup> may be independently: H, C <sub>1</sub> -C <sub>4</sub> alkyl sulphonate, C <sub>1</sub> -C <sub>4</sub> hydroxyalkyl sulphonate; C <sub>1</sub> -C <sub>4</sub> alkyl NHC (CH <sub>2</sub> OH) <sub>3</sub> , C <sub>1</sub> -C <sub>4</sub> alkyl, C <sub>1</sub> -C <sub>4</sub> hydroxyalkyl; C <sub>1</sub> -C <sub>4</sub> alkyl carboxylate; a polyelectrolyte; a buffer; and one or more additional components for example divalent metal salts.		

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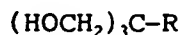
## STABILISATION OF PROTEINS IN SOLUTION

This invention relates to stabilisation of proteins in solution, particularly but not exclusively to stabilisation of enzymes. Alternative proteins include antibodies, antigens, serum compliment, vaccine components and bioactive peptides.

Use of enzymes in analytical applications has become well known because enzymes afford a number of significant advantages over conventional analytical chemistry. Enzymes confer specificity, sensitivity and operate under mild analytical conditions. A major disadvantage of enzyme based assays is that the enzyme component is often unstable. This may lead to degeneration of the reagent during storage and spurious results. Various methods have been used to increase the stability of enzymes including immobilisation, chemical modification by cross-linking, polymer grafting or substitution reactions, physical entrapment or encapsulation in polymer matrices or membranes and the addition of chemicals or solvents to the enzyme preparation. Enzyme preparations for use in analytical methods are often supplied in a dry stabilised form using a combination of chemical additives to promote stability. WO90/05182 and WO91/14773 disclose stabilisation of enzymes on drying by mixing aqueous solutions of the enzyme with soluble polyelectrolytes and cyclic polyols before removal of water from the solution. Such compositions have not been found to afford significant stabilisation prior to dehydration.

According to a first aspect of the present invention a protein stabiliser additive comprises two or more of:

- a. a tris(hydroxymethyl)methyl compound of formula 1;



(1)

wherein R is:  $\text{C}_1\text{-C}_4$  alkyl, substituted  $\text{C}_1\text{-C}_4$  alkyl,  $\text{NH}_2$ ;  $\text{NR}^1\text{R}^2$

wherein  $\text{R}^1$  and  $\text{R}^2$  may be independently: H,  $\text{C}_1\text{-C}_4$  alkyl sulphonate,  $\text{C}_1\text{-C}_4$  hydroxyalkyl sulphonate;  $\text{C}_1\text{-C}_4$  alkyl-NHC(CH<sub>2</sub>OH)<sub>3</sub>,  $\text{C}_1\text{-C}_4$  alkyl,  $\text{C}_1\text{-C}_4$  hydroxyalkyl;  $\text{C}_1\text{-C}_4$  alkyl

carboxylate;

- b. a polyelectrolyte;
- c. a buffer; and
- d. one or more additional components.

Component (a) may be referred to as a "tris" compound.

Examples of "tris" compounds include: 1,1',1"-tris(hydroxymethyl)ethane; 1,1',1"-tris(hydroxymethyl)propane; tris(hydroxymethyl)aminomethane or salts thereof for example chloride, maleate, phosphate, succinate salts; 1,3-bis[tris(hydroxymethyl)methylamino]propane; bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; N-[tris(hydroxymethyl)methyl]-2-aminoethane sulphonate; N-[tris(hydroxymethyl)methyl]-3-aminopropane sulphonate; N-[tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropane sulphonate; N-[tris(hydroxymethyl)methyl]-glycine.

The polyelectrolyte may be a cationic or anionic polyelectrolyte. Amphoteric polyelectrolytes may also be employed. The cationic polyelectrolyte is preferably a polymer with cationic groups distributed along the molecular chain. The cationic groups, which are preferably quaternary ammonium derived functions, may be disposed in side groups pendant from the chain or may be incorporated in it. Examples of cationic polyelectrolytes include: Copolymers of vinyl pyrrolidone and quaternary methyl methacrylate eg Gafquat series (755N, 734, HS-100) obtained from ISP; substituted polyacrylamides; polyethyleneimine, polypropyleneimine and substituted derivatives; polyamine homopolymers (Golchem CL118); polyamine co-polymers (eg condensates of epichlorohydrin and mono or dimethylamine); polydiallyl dimethyl ammonium chloride (polyDADMAC); substituted dextrans; modified guar gum (substituted with hydroxypropyltrimonium chloride); substituted proteins (eg quaternary groups substituted on soya protein and hydrolysed collagen); polyamino acids (eg polylysine); low molecular weight polyamino compounds (eg spermine and spermidine). Natural or artificial polymers may be employed.

Cationic polyelectrolytes with MW 150 to 5,000,000, preferably 5000 to 500,000, more preferably 5000 to 100,000 may be

employed. An amount of 0.01 to 10% is preferred, more preferably 0.1 to 2% w/v, especially 0.05 to 5%.

The anionic polyelectrolyte is preferably a polymer with anionic groups distributed along the molecular chain. The anionic groups, which may include carboxylate, sulphonate, sulphate or other negatively charged ionisable groupings, may be disposed upon groups pendant from the chain or bonded directly to the polymer backbone. Natural or artificial polymers may be employed.

Examples of anionic polyelectrolytes include: Gantrez (S-series, AN-series); alginic acid and salts; carboxymethyl celluloses and salts; substituted polyacrylamides (eg substituted with carboxylic acid groups); polyacrylic acids and salts; polystyrene sulphonic acids and salts; dextran sulphates; substituted saccharides eg sucrose octosulphate; heparin. Anionic polyelectrolytes with MW of 150 to 5,000,000 may be used, preferably 5000 to 500,000, more preferably 5000 to 100,000. An amount of 0.01% to 10% is preferred especially 0.05 to 5% more especially 0.1 to 2% w/v.

The said further component may be selected from the group comprising divalent metal ions, chelators for example EDTA, EGTA or citrate (not with peroxidases) or polyols. Preferred divalent metals include calcium and magnesium salts. Cobalt, zinc or manganese salts may also be employed.

The polyols which may be employed are preferably low molecular weight polyols although polymeric derivatives may be employed. Preferred polyols lower the dielectric of the solution. Such polyols include ethylene glycol, glycerol, erythritol and mannitol. Cyclic polyols which may be employed incorporate one or more alicyclic rings and may have at least one side chain. Preferred cyclic polyols include disaccharides and sugar alcohols, for example lactitol, sorbitol and inositol. Compounds having 2 to 10 hydroxyl groups are preferred. The amount of the polyol may be in the preferred range 1 to 5% more preferably 1 to 20% most preferably 2 to 10% w/v.

Compositions of the present invention stabilise enzymes

or other proteins without covalent or otherwise irreversible binding to the latter. The enzymes may be recovered intact from the solution by simple physical means, for example by adjustment of pH to a suitable value followed by salt or solvent precipitation, conveniently with ammonium sulphate.

Compositions of the present invention preferably consist essentially of one or more enzymes or other proteins together with buffers and stabilisers as described in the specification. Naturally occurring complex mixtures such as plasma, serum or other physiological fluids, which may include polyelectrolytes, hydroxy compounds and salts are excluded from the present invention. However immobilised, cross-linked, entrapped or covalently linked proteins are included within the present invention.

Compositions of the present invention are considered to stabilise a protein if the activity of the protein is not significantly diminished after a period of incubation at elevated temperatures in comparison to the protein in the absence of the stabilisers. For example horseradish peroxidase incubated at 60°C for 120 minutes shows no activity loss with stabilisers of this invention compared to 50% activity loss at 18 minutes under the same conditions.

According to a second aspect of the present invention a method of stabilising a protein includes the step of contacting the protein with an aqueous solution of an additive as described above.

According to a third aspect of the present invention there is provided use of an additive in accordance with the first aspect of this invention for stabilising an aqueous protein solution.

The present invention finds particular application in the preparation of analytical assay apparatus. A preferred aspect of the present invention provides an analytical assay formulation incorporating a stabiliser additive as defined above.

The invention is further described by means of example but not in any limitative sense.

The stability of protein solutions in the presence of stabilisers at elevated temperatures was investigated. Buffer solutions containing stabilisers were incubated to the required temperature in a Techne dry heating block. After several minutes incubation the temperature of the buffered mixture was measured using a thermistor. When the temperature was constant at the required level, protein solution was added and the tube was quickly inverted to mix thoroughly and returned to the dry block. Samples were taken at fixed time points thereafter and assayed for activity by standard procedures. All of the results were expressed as the amount of protein activity relative to the zero time activity. Zero time samples were obtained by incubation of the system at 25°C, duplicate samples being taken and assayed for protein activity

The present invention is demonstrated by reference to the data in Tables 1 to 20 which show relative specific activities of various proteins as a function of time. Solutions of the proteins without stabilisers underwent rapid denaturation as shown by loss of activity whereas with a polyelectrolyte present greater activity was retained for longer periods. Inclusion of one or more "tris" compounds gave a further increase in stability with the protein activity being retained. Tables 1 and 2 show the effects of a polyelectrolyte (Gafquat 755N), tris compounds and EDTA (as a metal chelator) on the solution stability of alcohol oxidase at 60°C.

Table 1. Effect of Stabilisers on Alcohol Oxidase Activity in Solution

Time (Min)	% Remaining Enzyme Activity				
	Control	Polyelectrolyte Alone	+Tris 89mM	+Tris ethane 89mM	+Tris propane 89mM
0	100.0	100.0	100.0	100.0	100.0
5	54.2	113.0	116.0	138.0	138.0
10	2.1	100.0	111.0	107.0	138.0
15	0	80.2	92.1	95.0	123.0
20	0	64.5	81.6	74.9	104.0
25	0	58.0	78.9	76.4	115.0
30	0	38.9	26.3	59.1	96.2

The buffer used in this example was 200mM phosphate pH 8.0.

Table 2. Effect of Tris and EDTA with Alcohol Oxidase

Time (Min)	% Remaining Enzyme Activity			
	Control	Polyelectrolyte Alone	+Tris 89mM and EDTA 1.99mM	+Tris no EDTA
0	100.0	100.0	100.0	100.0
5	61.0	113.0	162.0	116.0
10	15.3	100.0	168.0	111.0
15	10.2	80.2	154.0	92.1
20	6.8	64.5	134.0	81.6
25	6.8	58.0	140.0	78.9
30	5.1	38.9	123.0	26.3

The enzyme was alcohol oxidase from *Hansenula polymorpha* (50 units.ml<sup>-1</sup>). The tris solution was buffered to pH 8.0 with phosphoric acid.

The polyelectrolyte was Gafquat 755N (1%w/v).

The enzyme solution was thermally stressed at 60°C for 30



minutes with the recorded values being the percentage of remaining enzyme activity at 5 minute intervals during the incubation.

Table 3. Horseradish Peroxidase in 20mM Tris pH 8.0

Time (Min)	% Remaining Enzyme Activity			
	No Stabilisers	Gafquat 755N 0.5%w/v	Ethylene Glycol 10%v/v	Gafquat 755N 0.5w/v + Ethylene Glycol 10%v/v
0	100	100	100	100
5	77.6	88.1	72.6	90.8
10	69.1	76.2	63.4	78.1
15	57.8	63.5	57.1	70.75
20	48.1	59.3	52.5	63.4
25	46.0	53.6	48.5	59.25
30	39.3	48.9	43.8	55.1
40		46.5	41	50.7

Table 3 shows stabilisation of horseradish peroxidase in 20mM tris at pH 8.0, at a temperature of 60°C. The combination of the cationic polymer gafquat 755N and ethylene glycol produced better stabilisation than either components alone.

Table 4. Horseradish Peroxidase in 20mM Tris Buffer pH 8.0

Time (Min)	% Remaining Enzyme Activity			
	No Stabilisers	Gafquat 755N 0.5%	CaCl <sub>2</sub> 10mM	Gafquat 755N 0.5% + Ethylene Glycol 10% v/v + CaCl <sub>2</sub> 10mM
0	100	100	100	100
5	77.6	88.1	81.4	99.3
10	69.1	76.2	85.2	96
20	48.1	59.3	83.4	99.3
30	39.3	48.9	80.8	96
60			84.2	95
90			74.8	84.5
120			80.1	87.1
180			72.6	92.1
210			67.2	83.4
240				84.9

Table 4 shows stabilisation of horseradish peroxidase over an extended period at a temperature of 60°C. Calcium chloride alone produced good stabilisation but a combination of cationic polyelectrolyte, ethylene glycol and calcium chloride provided a high level of stabilisation for up to 240 minutes.

Table 5. Arthromyces Peroxidase

% Remaining Enzyme Activity					
Time (Min)	20mM Bis-Tris				
	Phosphate 20mM No Stabilisers	No Stabilisers	Gafquat 755N 0.5%w/v	CaCl <sub>2</sub> 10mM	Gafquat 755N 0.5%w/v + CaCl <sub>2</sub> 10mM
0	100	100	100	100	100
5	68.3	97.2			
10	63.4	92.6			
20		85.53	96	96.8	98.4
40	46.3	71.4	82	84	88.6
60			72	73.2	80.9
150			36	42	52

Table 5 shows stabilisation of arthromyces peroxidase at 4.5 Uml<sup>-1</sup> in 20 mM bis-tris at pH 7.3 at a temperature of 59°C. Stabilisation was obtained with the cationic polyelectrolyte gafquat 755N and also with calcium chloride. However superior stabilisation was obtained with a combination of both stabilisers.

Table 6. Pig Liver esterase. Gafquat/EDTA

% Remaining Enzyme Activity				
Time (Min)	No Stabilisers	Gafquat 0.5%w/v	EDTA 5mM	Gafquat 0.5%w/v EDTA 5mM
0	100	100	100	100
5	45.8	58.8	52.8	62.4
10	21.2	36	40.3	41.8
15	13.4	24	30	31.6
25	3.7	9.4	10	8.7
35		5.6		4.9

Table 6 shows stabilisation of pig liver esterase with gafquat and EDTA in 20 mM bis-tris pH 7.3 and an incubation temperature of 68.9°C.

Table 7. Pig Liver Esterase. Tris (Hydroxymethyl) ethane.

% Remaining Enzyme Activity

Time (Min)	No Stabilisers	Tris(OHMe) Et 1%	Tris(OHMe) Et 0.1%
0	100	100	100
5	45.8	60.5	65.1
10	21.2	26	39.6
15	13.4	15.2	26
25	3.7	4.6	10.2

Table 7 shows stabilisation of pig liver esterase in 20mM Bis-Tris pH 7.3 with tris (hydroxymethyl) ethane at 68.9°C.

Table 8. Stabilisation of Horseradish Peroxidase During Dilution in 20 mM Tris Buffer pH 8.0 at a Temperature of 25°C

% Relative Specific Activity

HRP-4 Concentration (ug/ml)	No Additive	CaCl <sub>2</sub> (10mM)	DEAE- Dextran MW 500K (0.5% w/v)	DEAE-Dextran MW 500K (0.5% w/v) + Ethylene Glycol (10%v/v) + CaCl <sub>2</sub> (10mM)
0.732	15	80	98	100
1.83	44	90	99	98
3.66	64	95	100	102
7.32	103	97	100	101
18.3	97	105	102	101
36.6	100	98	101	99

Table 8 shows the stabilisation of horseradish peroxidase in 20 mM tris at pH 8.0. DEAE-dextran both alone and in the presence of ethylene glycol and calcium chloride surprisingly afforded stability at extreme dilutions.

Table 9. The Stability of Dilute Solutions of Horseradish Peroxidase (3.66  $\mu\text{g/ml}$ ) At 37°C

% Remaining Enzyme Activity			
Time (Min)	No Stabilisers	DEAE-Dextran MW 500K (0.5%w/v)	DEAE-Dextran MW 500K (0.5% w/v) + Ethylene Glycol (10% v/v) + $\text{CaCl}_2$ (10 mM)
0	100	100	100
5	62	80	98
10	56	79	101
15	51	77	102
20	47	81	98
30	40	79	103
40	35	75	101
50	30	76	103
60	23	75	101
90		71	100
120		70	96
150		65	106
180		60	103

Table 9 shows stability of very dilute solutions of horseradish peroxidase (3.66  $\mu\text{g/ml}^{-1}$ ) at 37°C. A combination of DEAE-dextran, ethylene glycol and calcium chloride afforded excellent stabilisation for up to 180 minutes.

Table 10. Trypsin Autodigestion (Phosphate Buffer)

Time (Min)	% Remaining Enzyme Activity		
	No Stabilisers	Gafquat 755N 0.5%w/v	DEAE-Dextran 0.5%w/v
0	100	100	100
5	55.6	82.8	66.7
10	40.2	61.8	57.2
15	35.5	50.6	45.7
20	26.4	48.3	42.3
35			

Tables 10 and 11 show that the presence of polyelectrolyte retards autodegradation of trypsin, this effect was enhanced in the presence of tris stabiliser.

Table 11. Trypsin Autodigestion (Tris Buffer)

Time (Min)	% Remaining Enzyme Activity		
	No Stabilisers	Gafquat 755N 0.5%w/v	DEAE-Dextran 0.5%w/v
0	100	100	100
5	72.3	95	90.8
10	54.8	82.8	73.6
15	50.5	69	60.9
20	25.4	57.1	54.3
35	17.7	40	39.6

Table 12. Alkaline Phosphatase (Bovine)

Time (Min)	% Remaining Enzyme Activity	
	No Stabilisers	MgCl <sub>2</sub> 10mM Ethylene Glycol (10% v/v, 0.5% w/v) BSA and DEAE-Dextran (0.5 w/v)
0	100	100
5	61.4	88.5
10	45.7	72.5
15	42.1	66.4
20	36.8	61.9
40	30.1	50

Table 12 shows stabilisation of bovine alkaline phosphatase in 50 mM tris buffer at pH 8.0 and 61°C. Stabilisers comprising magnesium chloride, ethylene glycol, Bovine Serum Albumin and DEAE-dextran provided enhanced stabilisation.

**Table 13. The Effect of Combinations of Stabilisers on the Stability of Horseradish Peroxidase(Biozyme) Solutions (20 mM Tris/HCl Buffer pH 8.0) at 69°C**

**% Remaining Enzyme Activity**

Time (Min)	No Stabiliser	DEAE-Dextran MW 500K w/v)	Ethylene Glycol (10% v/v)	CaCl <sub>2</sub> (10 mM)	Ethylene Glycol (10% v/v) + CaCl <sub>2</sub> (10 mM)	DEAE-Dextran MW 500K w/v) + CaCl <sub>2</sub> (10 mM)	DEAE-Dextran MW 500K (0.5% w/v) + Ethylene Glycol (10% v/v) + CaCl <sub>2</sub> (10 mM)
0	100	100	100	100	100	100	100
5	78	88	94				
10	69	75	76				
15	58	67	64				
20	48	61	61	98	103	99	101
25	45	55	57				
30	39	50	53				
40				78	101	99	103
60				97	101	100	101
80				92	100	97	102
120				85	96	94	103
180				79	90	85	97
240						80	92



Table 13 shows the effect of combinations of stabilisers on the stability of horseradish peroxidase in 20 mM tris/HCl buffer at pH 8.0 and 69°C. This example shows the use of substituted dextrans as polyelectrolytes, a good stabilisation being obtained with DEAE-dextran, ethylene glycol and calcium chloride in combination.

Table 14. The Stability of HRP-4 (Biozyme) Solutions (20 mM Tris/HCl Buffer pH 8.0) at 50°C

% Remaining Enzyme Activity		
Time (Days)	No Stabiliser	DEAE-Dextran MW 500K (0.5% w/v) + Ethylene Glycol (10% v/v) + CaCl <sub>2</sub> (10 mM)
0	100	
0.0347	90.3	
0.056	95.1	
0.0764	90.3	
0.0972	82	
0.118	69.4	
0.139	65.4	
0.167	63.8	
0.26	46.3	
0.27	49.3	
0.29	38.4	101
1		
2		99
6		106
8		102.8

Table 14 shows stabilisation of horseradish peroxidase solutions in 20 mM tris/HCl buffer at pH 8 and 50°C. Degradation without stabiliser is rapid but good stabilisation was obtained at periods up to 8 days using a combination of DEAE-dextran, ethylene glycol and calcium chloride.

Table 15. The Effect of Temperature on the Stability of HRP-4 (Biozyme) Solutions (20 mM Tris/HCl Buffer pH 8.0) in the Presence of Ethylene Glycol (10% v/v), DEAE-Dextran MW 500K (0.5% w/v) and Calcium Chloride (10 mM)

Time (Min)	% Remaining Enzyme Activity			
	85.5°C	80.5°C	75.5°C	70.5°C
0	100	100	100	100
3	71			
5		80	91	
6	56			
9	39			
10		64	85	102
12	30			
15	28	53	82	
18	25			
20		47	77	100
21	22			
25		40		
30		33	73	103
40		30	68	
50		25	63	100
60			58	
70				99
110				100
130				93
150				93
170				88

Table 15 shows the effect of temperature on the stability of horseradish peroxidase solutions (20 mM tris/HCl buffer pH 8.0) in the presence of ethylene glycol, DEAE-dextran (MW 500K) and calcium chloride.

Table 16. Long Term Stability of HRP-4 Solutions

Time (Days)	% Remaining Enzyme Activity			
	No Stabiliser; 37°C	Stabilisation Buffer; 37°C	No Stabiliser; Room Temperature	Stabilisation Buffer; Room Temperature
0	100	100	100	100
6	90	101	96	99
15	85	98	93	100
21	76	99	86	101
33	32	99	82	101

Table 16 illustrates long term stabilisation of horseradish peroxidase solutions at different temperatures. The stabilisation buffer was the same as for example 15.

Table 17. The Stabilisation of HRP Activity of Antibody/HRP Conjugate (Sigma) Solutions (20 mM Tris/HCl Buffer pH 8.0) at 50°C

Time (Hours)	% Remaining Enzyme Activity		
	No Stabiliser	DEAE-Dextran MW 500K (0.5% w/v) + CaCl <sub>2</sub> (10 mM)	DEAE-Dextran MW 500K (0.5% w/v) + CaCl <sub>2</sub> (10 mM) + Ethylene Glycol (10% v/v)
0	100	100	100
0.5	86	99.4	100.6
20.8	35.9	80.1	100
48			99
72			97.6

Table 17 illustrates stabilisation of horseradish peroxidase activity of antibody-horseradish peroxidase conjugate solutions using the following stabiliser:

CaCl<sub>2</sub> 10mM, ethylene glycol 10% v/v, DEAE-dextran 0.5% w/v, Buffer Tris/HCl 20mM pH 8.0

The stabilisation of the HRP label of the IgG-HRP conjugate (Sigma A 6029) with the combination described

resulted in no loss of activity over 3 days incubation at 50°C.

Table 18. Galactose Oxidase: Stability at 66.5°C in 20mM Tris phosphate pH 7.84

% Remaining Enzyme Activity		
Time	No Stabiliser	Gantrez S-97 0.5%w/v
0	100	100
5	87.9	96.1
10	69.5	82.1
15	58.6	75.6
20	53.2	68

Table 19. Galactose Oxidase: Stability at 66.4°C in 20mM Tris phosphate pH 7.84

% Remaining Enzyme Activity		
Time	No Stabilisers	Sodium Alginate 0.2% w/v
0	100	100
5	87.9	114.5
10	69.5	102.8
15	58.6	99.8
20	53.2	79.5

Table 20. Alcohol Oxidase in 20 mM Bis-Tris pH 6.0

% Remaining Enzyme Activity		
Time	No Stabilisers	Carboxymethyl Cellulose 0.125%
0	100	100
5	15	37.1
10	10.7	27.9
20	8.9	21.8
30	7	19

## CLAIMS

1. A protein stabiliser additive comprising two or more of:

a. a tris(hydroxymethyl)methyl compound of formula 1;



wherein R is:  $\text{C}_1\text{-C}_4$  alkyl, substituted  $\text{C}_1\text{-C}_4$  alkyl,  $\text{NH}_2$ ;  $\text{NR}^1\text{R}^2$  wherein  $\text{R}_1$  and  $\text{R}_2$  may be independently: H,  $\text{C}_1\text{-C}_4$  alkyl sulphonate,  $\text{C}_1\text{-C}_4$  hydroxyalkyl sulphonate;  $\text{C}_1\text{-C}_4$  alkyl-NHC(CH<sub>2</sub>OH)<sub>3</sub>,  $\text{C}_1\text{-C}_4$  alkyl,  $\text{C}_1\text{-C}_4$  hydroxyalkyl;  $\text{C}_1\text{-C}_4$  alkyl carboxylate;

b. a polyelectrolyte;

c. A buffer; and

d. one or more additional components.

2. An additive as claimed in claim 1 wherein the tris(hydroxymethyl)methyl compound is selected from the group comprising: 1,1',1''-tris(hydroxymethyl)ethane; 1,1',1''-tris(hydroxymethyl)propane; tris(hydroxymethyl)aminomethane or salts thereof for example chloride, maleate, phosphate, succinate salts; 1,3-bis[tris(hydroxymethyl)methylamino]propane; bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; N-[tris(hydroxymethyl)methyl]-2-aminoethane sulphonate; N-[tris(hydroxymethyl)methyl]-3-aminopropane sulphonate; N-[tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropane sulphonate; N-[tris(hydroxymethyl)methyl]-glycine.

3. An additive as claimed in claim 1 or 2 wherein the polyelectrolyte is a cationic polyelectrolyte.

4. An additive as claimed in claim 3 wherein the cationic polyelectrolyte is a polymer with quaternary ammonium distributed along the molecular chain.

5. An additive as claimed in claim 3 or 4 wherein the cationic polyelectrolyte is selected from the group comprising copolymers of vinyl pyrrolidone and quaternary methyl methacrylate, substituted polyacrylamides, polyethyleneimine, polypropyleneimine and substituted derivatives thereof; polyamine homopolymers; polyamine copolymers; polydiallyl dimethyl ammonium chloride; substituted dextrans; modified guar gum; substituted proteins; polyamino acids and low molecular weight polyamino compounds including spermine and spermidine.

6. An additive as claimed in any of claims 3 to 5 wherein the cationic polyelectrolyte has a molecular weight of 150 to 5,000,000.

7. An additive as claimed in any of claims 3 to 6 wherein the cationic polyelectrolyte present in an amount of 0.01 to 10% w/v.

8. An additive as claimed in claim 1 or 2 wherein the polyelectrolyte is an anionic polyelectrolyte.

9. An additive as claimed in claim 8 wherein the anionic polyelectrolyte is selected from the group comprising alginic acids and salts; carboxymethyl celluloses and salts; substituted polyacrylamides; polyacrylic acids and salts; polystyrene sulphonate acids and salts; dextran sulphates; substituted saccharides; substituted oligosaccharides; substituted polysaccharides and heparin.

10. An additive as claimed in claim 8 or 9 wherein the anionic polyelectrolyte has a molecular weight of 150 to 5,000,000.

11. An additive as claimed in any of claims 8 to 10 wherein the anionic polyelectrolyte is present in an amount of 0.01 to 10% w/v.

12. An additive as claimed in any preceding claim wherein the further component is selected from the group comprising: divalent metal ions, chelators and polyols.

13. An additive as claimed in claim 12 wherein the further component is calcium or magnesium salt.

14. An additive as claimed in any preceding claim wherein the polyol is selected from the group comprising ethylene glycol, glycerol, erythritol and mannitol.

15. An additive as claimed in any preceding claim wherein the polyol is a cyclic polyol.

16. An additive as claimed in claim 15 wherein the polyol is selected from the group comprising lactitol, sorbitol and inositol.

17. An additive as claimed in any of claims 14 to 16 wherein the polyol is present in an amount of 0.1 to 25% w/v.

18. A method of stabilising a protein including the step of contacting the protein with an aqueous solution of an additive as claimed in any preceding claim.

19. Use of an additive as claimed in any of claims 1 to 17 for stabilising an aqueous protein.

## INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/GB 94/02180A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N9/96 C07K1/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	EP,A,0 190 041 (THE GREEN CROSS CORPORATION) 6 August 1986 see the whole document ---	1-19
X	US,A,4 282 316 (MODROVICH I.E.) 4 August 1981 see the whole document ---	1-19
A	GB,A,2 064 543 (SLOVENSKA AKADEMIA VIED, BRATISLAVA) 17 June 1981 see the whole document ---	1-19
X	WO,A,91 14773 (CRANFIELD BIOTECHNOLOGY LTD) 3 October 1991 cited in the application see the whole document ---	1-19
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 94/02180

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PCT/GB 94/02180

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